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Justin D. Crane *et al.*

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Editor's Summary

The Mechanism of Massage

Massage is a popular treatment—both for its putative healing properties and because it feels good—but is not among the usual tools of physicians. To validate its usefulness and understand how massage affects muscles in biomedical terms, Crane and his colleagues have documented the biological changes that massage evokes in the leg muscles of 11 young men who had just pushed themselves to exhaustion with heavy exercise. The exercise itself caused massive changes in gene expression, but after 10 min of massage, signaling pathways responsive to mechanical stresses were activated. Massage reduced signs of inflammation, and massaged muscle cells were better able to make new mitochondria —promoting faster recovery from exercise-induced muscle damage.

Massage stretches and pulls muscles and, as one might expect, the authors found that mechanosensory sensors focal adhesion kinase –1 and its downstream effectors extracellular signaling kinases 1 and 2 were activated, as revealed by their increased phosphorylation. Several hours after massage, another downstream target of this pathway, PGC-1α, shifted into the nucleus, where it in turn activated transcription of its own targets COX7B and ND1. This set of responses indicated that additional mitochondria were forming, presumably accelerating healing of the muscle. Massage also altered the behavior of NFκB, causing less of this key inflammatory mediator to accumulate in the nucleus. Consequently, the NF κB-regulated heat shock proteins and immune cytokines interleukin-6 and tumor necrosis factor –α were less active, a sign of less cellular stress and inflammation.

But one oft-repeated idea turned out not to be true. As others have shown, massage did not help clear lactic acid from tired muscles. And glycogen levels were also unchanged. Now that we know something about how massage heals, perhaps we will soon get some idea of why it is so enjoyable.

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MUSCLE

Massage Therapy Attenuates Inflammatory Signaling After Exercise-Induced Muscle Damage

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Massage therapy is commonly used during physical rehabilitation of skeletal muscle to ameliorate pain and promote recovery from injury. Although there is evidence that massage may relieve pain in injured muscle, how massage affects cellular function remains unknown. To assess the effects of massage, we administered either massage therapy or no treatment to separate quadriceps of 11 young male participants after exercise-induced muscle damage. Muscle biopsies were acquired from the quadriceps (vastus lateralis) at baseline, immediately after 10 min of massage treatment, and after a 2.5-hour period of recovery. We found that massage activated the mechanotransduction signaling pathways focal adhesion kinase (FAK) and extracellular signal–regulated kinase 1/2 (ERK1/2), potentiated mitochondrial biogenesis signaling [nuclear peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α)], and mitigated the rise in nuclear factor κB (NFκB) (p65) nuclear accumulation caused by exercise-induced muscle trauma. Moreover, despite having no effect on muscle metabolites (glycogen, lactate), massage attenuated the production of the inflammatory cytokines tumor necrosis factor–α (TNF-α) and interleukin-6 (IL-6) and reduced heat shock protein 27 (HSP27) phosphorylation, thereby mitigating cellular stress resulting from myofiber injury. In summary, when administered to skeletal muscle that has been acutely damaged through exercise, massage therapy appears to be clinically beneficial by reducing inflammation and promoting mitochondrial biogenesis.

INTRODUCTION

Complementary and alternative medicine (CAM) is increasingly used as a cost-effective adjunct to conventional medical care (*1*). Many CAM techniques, such as acupuncture, massage therapy, or chiropractic manipulations, are aimed at managing pain, relieving stress, and preventing injury. However, to date, most of these treatments have been evaluated only subjectively, and few have garnered sufficient cellular or mechanistic evidence of efficacy that validates their continued use on human patients.

Massage therapy is a well-known form of alternative medicine that consists of physical manipulation of muscle and connective tissue at a site of injury, inflexibility, or soreness to reduce pain and promote recovery (*1*, *2*). Massage has been hypothesized to moderate inflammation, improve blood flow, and reduce tissue stiffness, resulting in a diminished sensation of pain. The potential benefits of massage could be useful to a broad spectrum of individuals including the elderly, those suffering from musculoskeletal injuries, and patients with chronic inflammatory conditions. About 18 million individuals undergo massage therapy annually in the United States, making it the fifth most widely used form of CAM (*1*). The functional benefits of massage remain contentious in humans (*2*–*4*), and experiments using massage therapy in animals may not properly mimic the human responses, limiting their usefulness. Despite several reports that long-term massage therapy reduces chronic pain and improves range of motion in clinical trials (*5*–*7*), the biological effects of massage on skeletal muscle tissue remain unclear.

Muscle inflammation and pain are typically present when damage to the myofibrillar structure has occurred. This trauma, induced by either contractions (exercise-induced) or contusion, initially results in an inflammatory phase characterized by immune cell activation and infiltration, as well as cytokine release from both muscle and adjacent immune cells (*8*). A number of regulatory proteins are critical mediators of this stage of inflammation and cell repair, including those in the immune-responsive nuclear factor κB (NFκB) family as well as the transcriptional coactivator peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α), which is responsible for mitochondrial biogenesis (*9*, *10*). Repression of NFκB activation improves tissue repair and reduces immune cell infiltration into muscle (*9*, *11*). Ablation of muscle PGC-1α expression results in greater cytokine production and a state of low-grade inflammation (*10*), whereas increased PGC-1α reduces inflammation and improves the contractile function of dystrophic muscle (*12*). Furthermore, direct injection of the cytokines interleukin-6 (IL-6) and tumor necrosis factor–α (TNF-α) can induce pain and reduce muscle function, independent of any muscle damage (*13*, *14*). Therefore, moderating the activation of NFκB signaling, increasing the expression of PGC-1α, and attenuating cytokine release are all potential therapeutic approaches for treating inflammatory conditions in skeletal muscle.

In order for a mechanical stimulus such as stretch or strain to result in cell signaling, physical alterations in the cell membrane and the extracellular matrix must be transmitted via intermediate activating proteins known as integrins (*15*). Integrins in turn activate several intracellular kinases that propagate mechanotransduction signals, such as focal adhesion kinase (FAK) and the mitogen-activated protein kinase (MAPK) family of proteins. Skeletal muscle is sensitive to several types of stretch (*16*, *17*), and upon stretch activation, these kinase cascades activate regulatory factors that modulate protein synthesis, glucose uptake, and immune cell recruitment (*18*–*20*). Any physiological benefits due to massage would likely be initiated through mechanical

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effects on skeletal muscle followed by changes to intracellular regulatory cascades. The objective of this study was to assess the influence of massage within muscle that had performed a bout of intense exercise to better understand whether massage is an appropriate therapy for tissue rehabilitation. We obtained muscle biopsies from 11 young male subjects at rest, immediately after administration of massage to a randomized, single leg and after a 2.5-hour period of recovery (Fig. 1), and used whole-genome microarrays to screen for expressed genes induced by massage. After identifying functional categories from the array, we performed targeted real-time reverse transcription–polymerase chain reaction (RT-PCR), protein signaling analysis, and metabolite quantification to more completely characterize the processes within skeletal muscle that are influenced by massage.

RESULTS

Whole-genome expression profiling

Because of the paucity of studies that describe the cellular processes that are influenced by massage, our initial analysis used an untargeted gene profiling approach. We identified five genes that were differentially expressed in the muscle immediately after the massage treatment (0 hours) and four genes that were differentially expressed 2.5 hours after massage (Table 1). Independent of the massage treatment, the control leg muscle exhibited a change in 943 probes (representing 592 genes) at 0 hours after massage (30 min after exercise) and 2307 probes (representing 1309 genes) at 2.5 hours after massage (3 hours after exercise), significant changes that were induced by exercise alone (tables S1 and S2). A genome-wide analysis of the genes altered by exercise alone has been previously published by our group (*21*). One of the five genes whose expression was altered by massage immediately after the treatment was functionally related to actin dynamics (filamin B, β) (Table 1). One of the four genes induced by massage after recovery from treatment (2.5 hours) was related to NFκB nuclear trafficking (nucleoporin 88) (Table 1). Overall, this profile suggested that massage altered processes related to the cytoskeleton and to inflammation, with

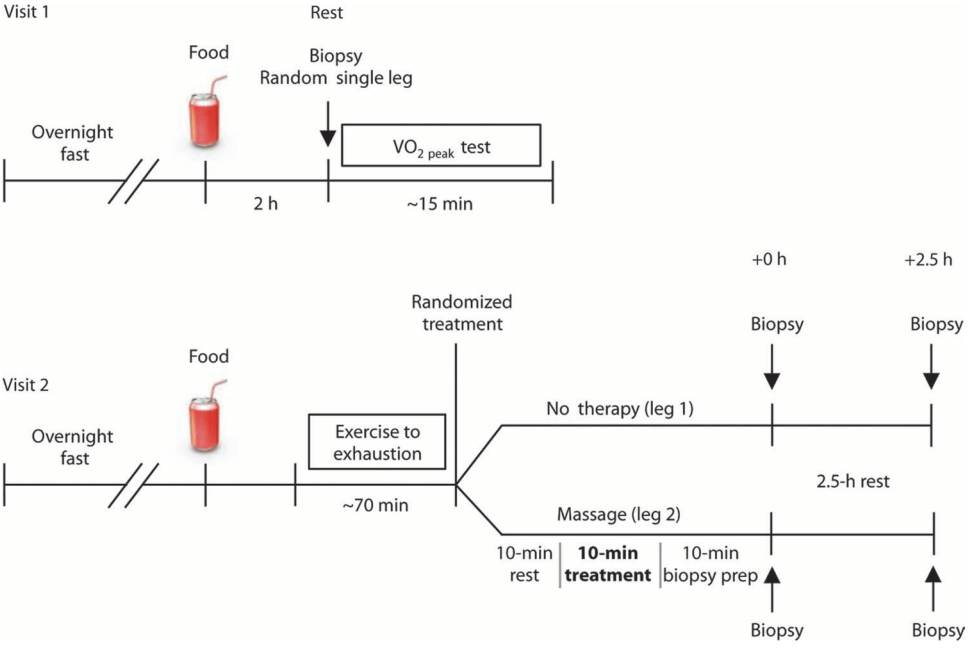

Fig. 1. Overview of the study trials and procedures.

Table 1. Genes with altered expression induced by massage therapy. Probes were significantly different ($P < 0.05$) in the massaged leg versus control at each time point after global gene expression analysis. $n = 11$ per time point.

Name	Probe	Gene	Fold change (versus control)	Function
		Immediately after massage (0 hours)		
Filamin B, β (actin-binding protein 278)	CR749793P06586	CR749793	1.03	Joining actin filaments
None	XM_929196P00253	XM_929196	1.68	Unknown
None	AK057762P01128	AK057762	0.84	Unknown
None	NM_014588P01652	NM_014588	1.27	Unknown
None	AK097340P00851	AK097340	1.25	Unknown
		2.5 hours after massage		
Stearoyl–coenzyme A desaturase 5	NM_001037582P01948	NM_001037582	0.73	Fatty acid synthesis
Misato homolog 1 (*Drosophila*)	BC002535P01465	BC002535	0.75	Unknown
Nucleoporin 88 kD	NM_002532P01539	NM_002532	1.48	Nucleocytoplasmic transport of NFκB
None	XM_208563P00344	XM_208563	0.81	Unknown

the former process being activated early after massage and the latter induced later in recovery.

Muscle damage and activation of mechanical signaling

To ensure that massage had been administered to muscle that was exposed to exercise-induced trauma, we confirmed that acute exercise had induced damage in skeletal muscle (number of disrupted fibers per square millimeter) compared to rest at both the 0- and the 2.5-hour time points (Fig. 2A, $P < 0.05$). Disrupted fibers identified by toluidine blue staining are indicative of z-disc streaming between adjacent myofilaments, revealing that tears to the muscle structure had occurred.

Mechanotransduction occurs rapidly in muscle in response to several types of stretch. Therefore, we assessed FAK and several of the MAPK proteins because they are the predominant signaling proteins that mediate mechanotransduction in skeletal muscle. We found that muscle from the massaged leg had greater FAK and ERK1/2 (extracellular signal–regulated kinase 1/2) phosphorylation immediately after treatment (Fig. 2, B to D) ($P < 0.05$). The immediate activation of these proteins suggested that they could play a role in transmitting the mechanical stimulus of massage therapy within skeletal muscle.

Growth signaling and metabolite concentrations

Mechanical manipulation of muscle can produce marked metabolic responses. Isolated stretch of skeletal muscle activates the phosphatidylinositol 3-kinase (PI3K) pathway (and the aforementioned MAPKs), induces growth, and enhances glucose uptake (*19*, *22*). However, Akt (Ser^{473}), mammalian target of rapamycin (mTOR) (Ser^{2448}), glycogen synthase kinase–3α (GSK-3α) (Ser^{21}), and GSK-3β (Ser^{9}), indicators of PI3K pathway activation, were unchanged by massage at either time point after treatment (Fig. 3, A to D) ($P > 0.05$). Moreover, there were no effects on muscle lactate levels (Fig. 3E) ($P > 0.05$), and proglycogen, macroglycogen, and total glycogen fractions were unaffected by massage treatment (Fig. 3, F to H) ($P > 0.05$). Therefore, the effects of ex vivo or in vitro stretch on growth or metabolite signaling within muscle were not mirrored by massage treatment.

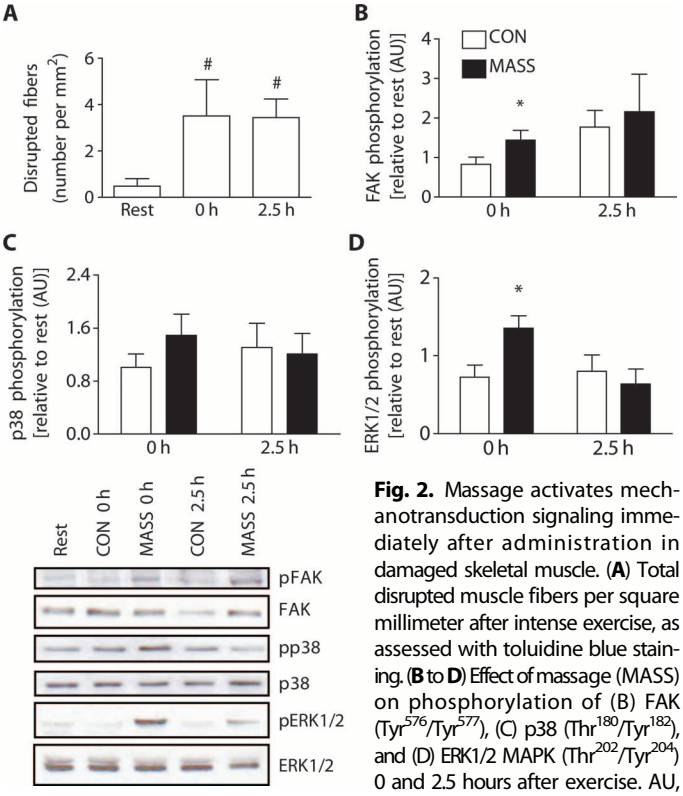

Fig. 2. Massage activates mechanotransduction signaling immediately after administration in damaged skeletal muscle. (**A**) Total disrupted muscle fibers per square millimeter after intense exercise, as assessed with toluidine blue staining. (**B** to **D**) Effect of massage (MASS) on phosphorylation of (B) FAK (Tyr^{576}/Tyr^{577}), (C) p38 (Thr^{180}/Tyr^{182}), and (D) ERK1/2 MAPK (Thr^{202}/Tyr^{204}) 0 and 2.5 hours after exercise. AU, arbitrary units. Representative Western blots are shown. $^{\#}P < 0.05$, significant difference from rest; $^{*}P < 0.05$, significant difference from control (CON). Data are expressed as means ± SEM. $n = 8$ to 11 per time point.

Mitochondrial biogenesis

Our observation that MAPK-related signaling proteins were activated by massage then led us to consider downstream targets of this cascade. PGC-1α is an important mediator of tissue repair and metabolic control, and its expression is increased by FAK and the MAPK p38 (*23*, *24*). In accordance with our gene expression results, the nuclear abundance of PGC-1α was higher in the massaged leg 2.5 hours after treatment (Fig. 4A, $P < 0.05$) but not immediately after massage ($P > 0.05$). The expression of COX7B and ND1 mRNA, mitochondrial electron transport chain components encoded by nuclear and mitochondrial genes, respectively, was also assessed, because they are transcriptionally activated by PGC-1α. Similar to the results for nuclear PGC-1α protein, COX7B and ND1 mRNA were increased at 2.5 hours in the massaged leg ($P < 0.05$) but not immediately after massage was administered (0 hours, $P > 0.05$), confirming that mitochondrial biogenesis signaling was augmented by massage therapy (Fig. 4, B and C).

NFκB, cytokine, and heat shock protein signaling

The results of our genome profiling revealed massage-induced expression of a factor involved in NFκB transport into the nucleus (nucleoporin 88). Given the critical role of NFκB in muscle inflammation, we sought to assess its activation (via nuclear accumulation) and any downstream effects on cytokine production. The nuclear abundance of NFκB was reduced in the treated leg immediately after massage ($P < 0.05$) but not 2.5 hours later ($P > 0.05$) (Fig. 5A). Because the activation of heat shock proteins (HSPs) is indicative of cellular stress and these protein chaperones can be up-regulated by NFκB signaling, we also determined their response to massage. In agreement with our NFκB results, the phosphorylation of HSP27 (Ser^{82}) was reduced in the massaged leg compared to the control at 2.5 hours ($P < 0.05$) but not at 0 hours ($P > 0.05$) (Fig. 5B). IL-6 protein content, a downstream response of NFκB activation, was reduced in the massaged leg at 2.5 hours ($P < 0.05$) but was not different from the control leg at 0 hours ($P > 0.05$) (Fig. 5C). In addition, the proportion of mature TNF-α to precursor TNF-α protein was lower at 0 hours with massage than with no treatment ($P < 0.05$) but not at 2.5 hours (Fig. 5D). TNF-α is only soluble and free from the cell membrane when it has been processed to its mature form (*25*).

In contrast to the effects of massage on HSP27, no changes were induced in the abundance of the cytosolic chaperone HSP70, nor in the mitochondrial HSPs GRP75 or HSP60, by massage (Fig. 6, $P > 0.05$).

DISCUSSION

The effectiveness and mechanistic underpinnings of movement or touch-based rehabilitation medicine (physiotherapy) and its related

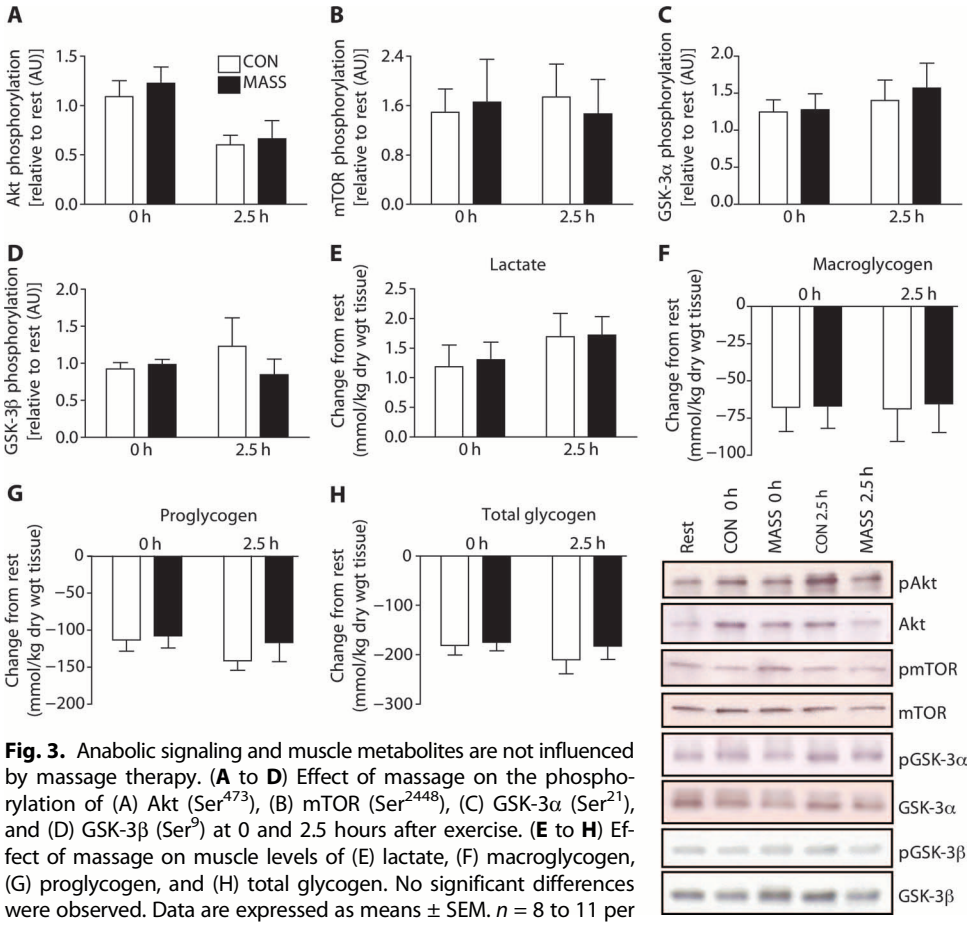

Fig. 3. Anabolic signaling and muscle metabolites are not influenced by massage therapy. (**A** to **D**) Effect of massage on the phosphorylation of (A) Akt (Ser^{473}), (B) mTOR (Ser^{2448}), (C) GSK-3α (Ser^{21}), and (D) GSK-3β (Ser^{9}) at 0 and 2.5 hours after exercise. (**E** to **H**) Effect of massage on muscle levels of (E) lactate, (F) macroglycogen, (G) proglycogen, and (H) total glycogen. No significant differences were observed. Data are expressed as means ± SEM. $n = 8$ to 11 per time point.

techniques are largely unsubstantiated. The increasing use of massage therapy as an adjunct to conventional care for musculoskeletal injury recovery (*1*) and the growing number of physician referrals for massage (*26*) represent a shift toward non–drug-based therapies for personal health. Given the spiraling cost of primary care and medications in the United States, it is likely that more patients will seek out this therapy as well as other nontraditional medical alternatives to complement more conventional approaches to their healthcare. In particular, because musculoskeletal problems have a significant impact on daily function and quality of life, it is important to validate treatments that enhance recovery, moderate inflammation, and reduce pain in skeletal muscle. To begin this process, we have assessed the molecular influence of massage on human muscle cells and determined that muscle damaged by exercise is responsive to a 10-min session of massage. After activating cellular signaling pathways through mechanotransduction, massage attenuated the rise in several other signaling pathways indicative of muscle inflammation and cell stress regulated by NFκB and also augmented signaling through PGC-1α.

Our results are likely at least partly due to mechanical stretch or strain during massage treatment that activated mechanotransduction signaling via the FAK and ERK1/2 signaling pathways. These proteins were phosphorylated immediately after massage treatment, and their activation preceded several signaling events that were activated later. Isolated stretch increases muscle glucose uptake, protein synthesis, and, ultimately, muscle growth through activation of the MAPK and PI3K cascades (*19*, *22*, *27*, *28*). In contrast to these results, we did not detect any acute changes in Akt phosphorylation or its downstream targets mTOR, GSK-3α, or GSK-3β after massage. In addition, we did not observe any alterations in muscle glycogen levels nor in muscle lactate, suggesting that the acute effects of massage occur independent of PI3K signaling, glucose uptake, or lactate clearance. Muscle responds to stretch differently depending on the loading axis (*16*, *17*), and previous studies demonstrating PI3K activation have typically used only axial stretch (*19*, *27*), often at supraphysiologic levels, which may produce different signals within the muscle than those resulting from massage. Our results demonstrate that muscle remains mechanically sensitive to 10 min of massage therapy even after exhaustive exercise, which likely potentiates cell signaling via PGC-1α and NFκB.

PGC-1α governs a host of cellular functions, primarily those that enhance metabolism and increase mitochondrial content, and is postulated to be an important, indirect mediator of tissue inflammation in skeletal muscle (*29*). Metabolic fitness is intrinsically linked to cellular stress resistance, and, accordingly, low expression of PGC-1α has been tied to greater inflammatory cytokine expression (*10*, *30*). The regulation of inflammation is typically considered separate from mitochondrial biogenesis; however, the transcription factor NFκB (p65) has been recently shown to bind directly to PGC-1α in cardiomyocytes (*31*), suggesting a mechanism by which these important pathways may interact. Our data agree with these findings, because the immediate reduction in nuclear NFκB (p65) that we observed in the massaged leg preceded the rise in nuclear PGC-1α at 2.5 hours, possibly through decreased direct binding of p65. Thus, massage may lower the amount of p65 available to sequester PGC-1α in the nucleus under conditions of exercise-induced muscle damage, indirectly favoring metabolic recovery.

Acute, contraction-induced damage to skeletal muscle is known to activate the inflammatory NFκB pathway, triggering the shuttling of the p65 subunit into the nucleus, which increases prostaglandin synthesis, acute phase proteins, and inflammatory cytokine expression (*32*, *33*). In our study, the reduced nuclear abundance of the p65 subunit of NFκB after massage correlated with a reduction in the ratio of mature to precursor TNF-α protein immediately after massage and a reduction in IL-6 2.5 hours later, consistent with attenuated local production of cytokines. Inflammatory cytokines may impede muscle repair by increasing muscle protein breakdown (*34*–*36*) and suppressing myosin synthesis (*37*). Additionally, their local injection activates peripheral nociceptors, causing increased sensitivity to pain (hyperalgesia) (*13*, *14*). Administration of cyclooxygenase inhibitors blunts most of

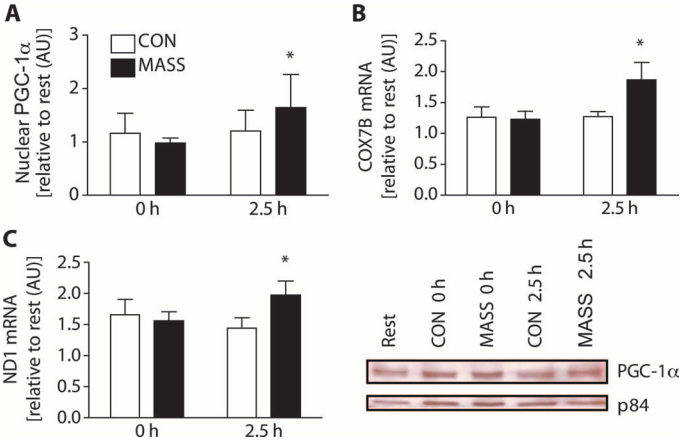

Fig. 4. Nuclear PGC-1α and levels of mitochondrial mRNA are increased 2.5 hours after massage therapy. (**A**) Effect of massage on nuclear PGC-1α protein abundance as determined by Western blotting. (**B** and **C**) Effect of massage on mRNA levels of nuclear-encoded COX7B (B) and mRNA levels of mitochondria-encoded ND1 (C), as measured by RT-PCR. *$P < 0.05$, significant difference from control. Data are expressed as means ± SEM. $n = 5$ to 8 for nuclear proteins and $n = 10$ to 11 for mRNA expression.

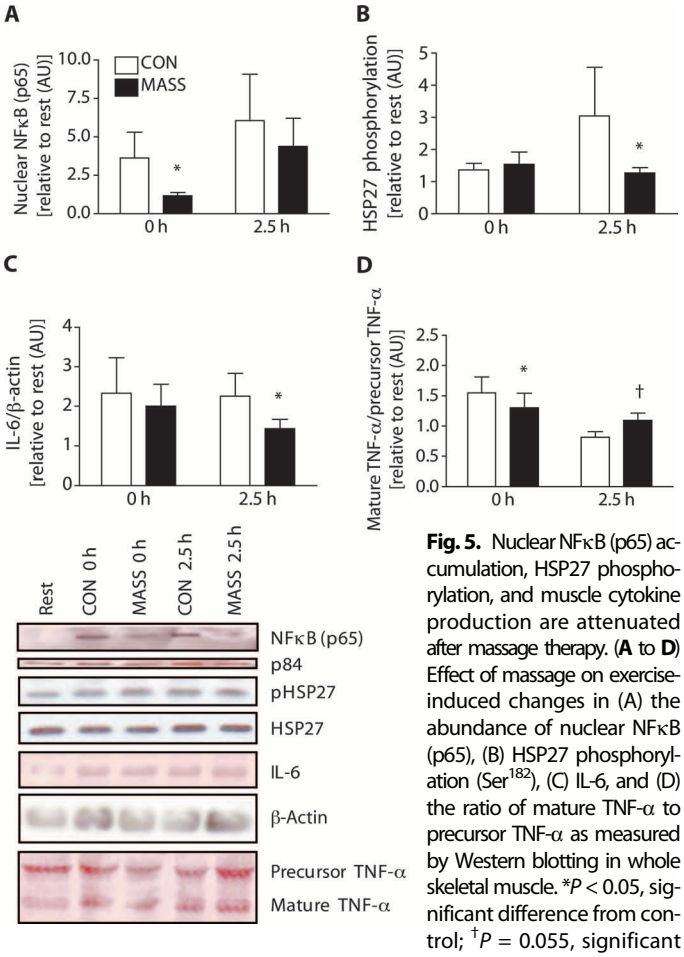

Fig. 5. Nuclear NFκB (p65) accumulation, HSP27 phosphorylation, and muscle cytokine production are attenuated after massage therapy. (**A** to **D**) Effect of massage on exercise-induced changes in (A) the abundance of nuclear NFκB (p65), (B) HSP27 phosphorylation (Ser^{182}), (C) IL-6, and (D) the ratio of mature TNF-α to precursor TNF-α as measured by Western blotting in whole skeletal muscle. *$P < 0.05$, significant difference from control; †$P = 0.055$, significant difference from control. Data are expressed as means ± SEM. $n = 5$ to 8 for nuclear proteins and $n = 7$ to 11 for whole muscle proteins.

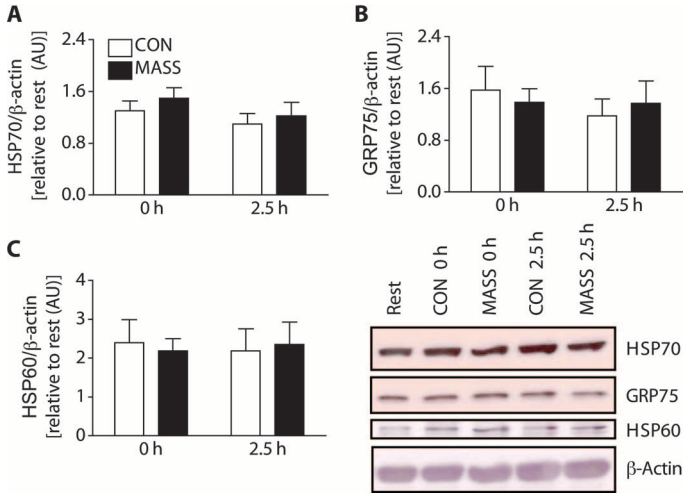

Fig. 6. Large mitochondrial and cytosolic HSPs do not change in response to massage. (**A** to **C**) Influence of massage on the abundance of (A) HSP70, (B) GRP75, and (C) HSP60 as determined by Western blotting in whole skeletal muscle. No significant differences were observed. Data are expressed as means ± SEM. $n = 8$ to 11 per time point.

the hyperalgesia derived from local IL-6 and TNF-α injection in rodent muscle, indicating that prostaglandins are largely responsible for the sensitization of muscle-associated nerves caused by inflammatory cytokines (*38*, *39*). Similarly, pain and inflammation in human patients are often treated with analgesic medications that block the local formation of prostaglandins (*40*), suggesting that massage may act in a similar fashion. One class of analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs), are some of the most commonly consumed drugs in the world, but under certain situations, side effects, interactions with other medications, or preexisting conditions may preclude their use. An alternative therapy such as massage may provide similar benefits. Moreover, massage may be useful in situations where areas of low blood flow (the muscle tendon interface) restrict the access of circulating analgesics to a site of inflammation or in conjunction with other anti-inflammatory treatments.

In summary, our findings suggest that the perceived positive effects of massage are a result of an attenuated production of inflammatory cytokines, which may reduce pain by the same mechanism as conventional anti-inflammatory drugs such as NSAIDs. These results elucidate the biological effects of massage in skeletal muscle and provide evidence that manipulative therapies may be justifiable in medical practice. Future studies should address additional posttranslational signaling pathways influenced by manual therapies (such as whole-proteome phosphorylation and acetylation), as well as the effect of chronic massage on skeletal muscle adaptations to exercise.

MATERIALS AND METHODS

Study description

Eleven healthy, recreationally active males volunteered to participate in this study. Subject characteristics are provided in table S3. Before subject recruitment, this study was approved by the McMaster University Research Ethics Board. After an explanation of the study procedures, all individuals gave their informed consent to participate in the investigation. Participants reported to the laboratory the morning

after an overnight fast on two separate occasions, separated by a 2-week period (see Fig. 1 for a schematic of the study procedures). They were requested to adhere to the following requests before each visit: abstain from moderate to intense physical exertion for 72 hours, refrain from alcohol consumption for 48 hours, eat their habitual diet for 48 hours, and abstain from caffeine for 12 hours. Participants were given a 355-kcal defined formula diet (Ensure, Ross Laboratories), which was consumed 2 hours before each study trial. At the beginning of the first visit, a baseline muscle biopsy was acquired from the vastus lateralis of a randomly assigned leg to serve as the resting control sample (Rest). After the biopsy, each subject underwent testing for peak aerobic capacity (VO_{2peak}) on an upright cycle ergometer as described (*21*). At the second visit, all subjects returned to the laboratory and performed a bout of exhaustive aerobic exercise, as described (*21*), before receiving randomized massage treatment. The exercise bout consisted of upright cycling exercise on an electrically braked cycle ergometer (Lode Excalibur, Lode) pedaling at a workload calculated to elicit 60% of their predetermined VO_{2peak} for 30 min at a cycling cadence between 70 and 90 rpm. After 30 min, the intensity was increased to a workload equivalent to 65% VO_{2peak} for 5 min, then dropped back to 60% for 5 min, increased to 70% VO_{2peak} for 5 min, dropped to 60% for 5 min, etc., to a maximum of 85% VO_{2peak}. If 85% VO_{2peak} was attained, then subjects continued with intervals of 85% VO_{2peak} for 2 min followed by 60% VO_{2peak} for 2 min, etc., until subject exhaustion. Test completion was ascertained when subjects were unable to maintain a cycling cadence above 70 rpm. We used acute aerobic exercise in unconditioned individuals to cause contraction-induced muscle damage to mimic a common scenario where massage therapy is used in human subjects. Immediately after exercise, subjects were allowed to recover for 10 min while massage oil was lightly applied to both quadriceps. Thereafter, a single leg was randomized to receive massage treatment for 10 min from a registered massage therapist.

The massage treatment was composed of three types of soft tissue manipulations while the subject remained in the supine position. Treatment was focused on the knee extensor muscles, encompassing a range of pressures and movement patterns typically provided during a therapy session. The treatment consisted of (i) 2 min of effleurage, a light stroking technique delivered with a moderate pressure; (ii) 3 min of petrissage, a firm motion involving compression and subsequent pressure release from the muscle; (iii) 3 min of slow muscle stripping, consisting of repeated longitudinal strokes of ~40 s; and (iv) an additional 2 min of effleurage. All members of the study team were blinded as to which leg was massaged, with the exception of the massage therapist. After massage, the subjects rested for 10 min and a muscle biopsy was obtained from the vastus lateralis of each leg (0 hours). Two and a half hours later (3 hours after the cessation of the exercise bout), a biopsy was again obtained from each leg (2.5 hours). All biopsies were acquired from separate incisions, about 2 to 3 cm apart. A small portion of muscle from each biopsy was fixed in chilled (4°C) 2% glutaraldehyde supplemented with 0.1% sodium cacodylate for subsequent histology. The remaining muscle samples were quickly portioned, flash-frozen in liquid nitrogen, and then stored at −86°C until further analysis.

Muscle metabolites

About 20 mg of muscle tissue was freeze-dried to remove all cellular water content, and metabolites were extracted serially with perchloric acid (PCA) and hydrochloric acid (HCl) as described (*41*). Using this extraction procedure, we quantitated weak acid–soluble (macroglycogen) and weak acid–insoluble (proglycogen) fractions, as well as total glycogen values, by comparing samples to a glucose standard curve (5 to 600 μM). Glycogen values were determined by monitoring the NADPH [reduced form of nicotinamide adenine dinucleotide (NAD^+) phosphate] formed from free glucose by hexokinase and glucose-6-phosphate dehydrogenase with appropriate substrates in a 1-ml cuvette as described (*42*). Lactate was analyzed in a 96-well plate using 10 μl of the PCA fraction by generating NADH (reduced form of NAD^+) from endogenous lactate in 185 μl of buffer containing 100 mM hydrazine, 100 mM glycine, and 0.5 mM NAD^+. Lactate dehydrogenase was then added at a concentration of 8 U/ml, followed by a 2-hour incubation, and samples were compared to a lactate standard curve (5 to 100 μM). This protocol is fundamentally as described (*42*). Both NADPH and NADH were monitored at an excitation of 340 nm and emission of 460 nm with a fluorometer (cuvette: Perkin-Elmer, plate reader: Tecan). All enzymes were acquired from Roche, and all chemicals were from Sigma-Aldrich.

RNA extraction, microarray analysis, and RT-PCR

RNA was extracted from whole muscle with an automated homogenization and extraction apparatus (QiaCube, Qiagen) according to the manufacturers' protocols for the RNeasy mini kit. RNA yield and integrity was then evaluated via Bioanalyzer (Agilent), and samples that passed QC and integrity checks were then reverse-transcribed and amplified with one round of whole-transcriptome amplification via the WTA2 Complete Whole Transcriptome kit from Sigma. After amplification, the resulting omniplex complementary DNA (cDNA) library was further purified with a Qiagen PCR purification chip and further evaluated for integrity and yield via Bioanalyzer (Agilent). cDNA libraries were then labeled, hybridized, and washed onto 12-plex 135K (HG18) NimbleGen oligonucleotide whole-genome microarrays (Roche NimbleGen), via the one-color NimbleGen labeling kit as per the manufacturer's instructions. The resulting hybridized chips were then scanned and quantitated via a GenePix 4200 Scanner (Molecular Devices), and the quantitated images were used to evaluate relative gene expression between samples.

After global genome profiling analysis, reverse transcription was performed on 200 ng of total RNA produced above with a High-Capacity Reverse Transcription kit (Applied Biosystems) on a gradient thermocycler (MyCycler, Bio-Rad). Real-time PCR was performed in duplicate with the 7300 Real-Time PCR system (Applied Biosystems) using SYBR Green chemistry (PerfeCTa SYBR Green Supermix with ROX, Quanta Biosciences) under standard thermocycling conditions. Genes of interest were normalized to the housekeeping gene β*2-microglobulin*, which was not influenced by massage treatment. Primer sequences used in the study are as follows: ND1, aagtcaccctagccatcattctac (forward) and gcaggagtaatcagaggtgttctt (reverse); COX7B, ctagcttcaccttcacgatgtt (forward) and gctctgccttgccattgt (reverse); β2-microglobulin, acttgtctttcagcaaggactg (forward) and ttcacacggcaggcatact (reverse). All primers were found to have >90% efficiencies by a standardized serial dilution curve, and a melt-curve analysis was performed to verify that a single transcript was produced.

Western blotting

Whole muscle was prepared for Western blotting as follows: A portion of muscle was homogenized on ice with a glass-on-glass homogenizer (Wheaton) in 25 times volume per weight (25 μl per 1 mg of tissue) of potassium phosphate buffer (50 mM K_2HPO_4/KH_2PO_4, 1 mM EDTA,

0.1 mM dithiothreitol, pH 7.4) supplemented with phosphatase and protease inhibitors (Roche). Homogenates were centrifuged at 600*g* to pellet the insoluble debris, and supernatants were transferred to clean tubes and stored at −86°C.

Nuclear protein extraction was performed as follows: Respective cellular fractions were isolated from ~20 mg of whole muscle with the NE-PER kit (Pierce) on ice with minor modifications to the manufacturer's protocol: (i) Muscle was briefly minced with scissors before homogenization to allow for a more complete dispersion of the muscle tissue in the buffer. (ii) Both the CERI and NER buffers were supplemented with protease and phosphatase inhibitors (Roche). (iii) The NER buffer was supplemented with 0.1% SDS to more fully dissociate the soluble protein fraction of muscle nuclei. Our lab has previously used this procedure to extract pure nuclear protein fractions (*43*). Nuclear proteins were pipetted into clean microcentrifuge tubes after the isolation procedure and stored at −86°C.

SDS–polyacrylamide gel electrophoresis

Protein concentration was determined in homogenates with the bicinchonic acid method as per the manufacturer's recommendations (Pierce) with a spectrophotometer (Benchmark Plus, Bio-Rad). Equivalent amounts of protein from each sample were combined with loading buffer and resolved on 7, 10, or 12.5% acrylamide gels depending on the molecular weight of the protein of interest. Proteins were separated at 120 V for about 2 hours and then transferred at 110 V for 1 hour to nitrocellulose membranes (GE Healthcare). Membranes were blocked with milk or bovine serum albumin diluted in tris-buffered saline with Tween (TBS-T) for 1 hour and then incubated in primary antibody overnight at 4°C. The manufacturer and antibody dilutions used are given in table S4. After primary incubation, blots were washed three times in TBS-T and incubated in anti-mouse or anti-rabbit secondary antibodies (1:10,000; GE Healthcare) at room temperature for 1 hour. Subsequently, all blots were developed with ECL Plus (GE Healthcare) and exposed to x-ray film (GE Healthcare). All films were digitized, and band density was determined with ImageJ (National Institutes of Health). Phosphoprotein antibodies were stripped for 1 to 2 hours at room temperature with Restore buffer (Pierce), reprobed with secondary antibody, and developed for at least 10 min to ensure that no signal was present. Blots were then probed with the respective total antibody. In cases where stripping was unsuccessful, phosphorylated and total proteins were normalized to a stable loading control (β-actin), and then phosphorylated-to-total protein ratios were established in separate blots. Nuclear protein extracts were normalized to p84, which was stable after massage treatment.

Histology

Glutaraldehyde-fixed muscle was postfixed in 1% osmium tetroxide, dehydrated in graded alcohol, and embedded in Spurr's resin. Longitudinal, semithin sections (~1 μm) were cut with a glass knife and stained with toluidine blue for light microscopic evaluation as described (*44*). Focal damage encompassed no more than two adjacent z discs, and extensive damage encompassed more than two adjacent z discs. We have previously confirmed the accuracy of toluidine blue to detect z-disk streaming (disruption of thick and thin muscle filament organization) by comparing this method to transmission electron microscopy images (*44*). Results are expressed as the total number of focal or extensive areas of z-disc streaming per fiber area (total disruptions per square millimeter).

Statistics

All data except for the genome array were analyzed with a paired *t* test between the massaged and the control leg at each time point, unless data were not normally distributed. When a data set failed to meet qualifications for a normal distribution, it was analyzed with a Wilcoxon matched-pairs nonparametric test. For a specific comparison, according to a priori hypotheses, where no differences were seen between treatments but the comparison to rest was still necessary (that is, to confirm muscle damage induction), control and massaged leg data were averaged at each time point and compared by *t* test individually to the resting data. Statistical tests were performed with Statistica 4.

For the genome array, to minimize nuisance experimental variation among the genomic data, we used between-array loess normalization with the *affy* package in the Bioconductor repository (*45*). We used a mixed-model approach (*46*) to estimate the differential expression (at the $\log_2$ scale) of massage. Specifically, we fit a model, for each probe separately, of the following form:

$$Y^g_{ijk} = \beta^g_{0,t} + \beta^g_{0i,t} + \beta^g_{1,t} I(\text{Time}_{ijk} = t) + \beta^g_{2,t} I(\text{Time}_{ijk} = t) \times I(\text{Massage}_{ij}) + e^g_{ijk},$$

where Y_{ijk} is the $\log_2$ expression for the *g*th probe, *i*th person, *j*th time, *k*th leg; $I(\text{Time}_{ijk} = t)$ is the indicator that the time of sample is post-baseline (either 30 min or 3 hours), and likewise $I(\text{Massage})$ is the indicator of whether the leg is the massage leg or not; $\beta^g_{0i,t}$ is a random effect at the individual level; and the other β are fixed coefficients (parameters). In this case, the parameter of interest is $\beta^g_{2,t}$, which estimates the mean $\log_2$ difference in expression of the massaged versus nonmassaged leg at time *t*. Thus, the *P* value, by probe (and time $t = 0$ min and 2.5 hours), is that for the null, $H_0 : \beta^g_{2,t} = 0$, where the Wald statistic is returned by the mixed-model procedure (*47*). As indicated, we performed these regressions by both probe and post-baseline time of measurement (0 min and 2.5 hours). For each of the sets by time, we adjusted for multiple testing using family-wise error rate (Holm's method) (*45*).

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/4/119/119ra13/DC1
Table S1. Control leg gene expression 30 min after exercise. (Excel file)
Table S2. Control leg gene expression 3 hours after exercise. (Excel file)
Table S3. Characteristics of the study participants.
Table S4. Primary antibodies used in immunoblotting.

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